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THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application For Renewal of Research Grant

(Use extra pages as needed)

First Renewal ☒ Second Renewal ☐ Date: 7-10-74

1. Principal Investigator (give title and degrees):

Jay A. Levy, M.D., Assistant Clinical Professor of Medicine, Department of Medicine and Research Associate, Cancer Research Institute

2. Institution & address:

University of California
San Francisco, California 94143

3. Department(s) where research will be done or collaboration provided:

Cancer Research Institute

4. Short title of study:

Development of a model system in vitro for studying carcinomas.
Study of possible interplay of endogenous viruses and chemicals in cocarcinogenesis.

5. Proposed renewal date: January 1, 1975

6. How results to date have changed earlier specific research aims:

Our inability to grow epithelial-like cells over the past three years indicates to us that the present procedures developed offer no new aid in obtaining well differentiated epithelial cells. Instead we plan to concentrate on the NZB liver cell line in looking at a possible in vitro model for carcinogenesis. The discovery of the universality of xenotropic virus has suggested that this C-type virus may play a role in chemical carcinogenesis. Our aim therefore is to study by in vitro and in vivo systems, the activation of this virus by chemicals and to correlate these events with the serologic and pathologic response of the host.

7. How results to date have changed earlier working hypothesis:

The results we have obtained so far have not changed any hypotheses we have had on the possible role of viruses in carcinogenesis. On the contrary, they have encouraged our studies in this field since it is clear that chemicals under certain conditions can lead to activation of viruses and perhaps dedifferentiation of normal cell function.

8. Any additional facilities now required? Describe briefly:

CO_2 incubator

Freezer and refrigerator

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

No

10. Append outline of experimental protocol for ensuing year.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

12. Summary progress report (append in standard form as separate document, unless recently submitted).

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested) (fringe benefits included)		
Levy, Jay A., Principal Investigator	30%	—
Technical		
Oleszko, Oksana, Staff Research Associate I	100	12,068
Ramirez, Michele, Secretary II	100	11,245
	Sub-Total for A	23,313
B. Consumable supplies (by major categories)		
Media and sera		10,500
Animals, purchase and maintenance		500
Glassware, pipettes, plastic flasks, petri dishes		10,500
CO_2 tanks and liquid nitrogen		1,000
Cell: mouse, rat, human		1,800
	Sub-Total for B	24,300
C. Other expenses (itemize)		
Travel -- domestic		800
Publication and photography expenses		800
	Sub-Total for C	1600
	Running Total of A + B + C	49,213
D. Permanent equipment (itemize)		
CO_2 incubator (NAPCO)		1,857
Freezer, cabinet, upright		1,040
Refrigerator, Lab Line		753
	Sub-Total for D	3,650
E. Indirect costs (15% of A+B+C)	E	7,382
	Total request	60,245

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14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Jay A. Levy, M.D.

Signature Jay A Levy Date 7-22-74

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Checks payable to

The Regents of the Univ. of Calif.

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Responsible officer of institution

Typed Name Sue Clark

Title Gifts & Endowments - Program Coordinator

Signature Sue Clark Date 7/26/74

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10. Proposal for continued studies

We would like to continue research centered primarily on the role xenotropic virus and chemicals may play in carcinogenesis. Both in vitro and in vivo studies are proposed.

A. In vitro experiments

In continual efforts to determine whether an epithelial cell line can be derived to provide a good in vitro model for testing chemical and viral carcinogenesis, clones of the NZB liver line will be further studied. The line has been sent to Dr. Kouri for quantitation of AHH activity. If these cells are making enzymes characteristic of liver epithelial cells and have inducible AHH activity, we shall use them for studies of chemical transformation. The cells will be treated with varying dilutions of methyl cholanthrene and observed for the formation of foci of cell alteration. Foci will be isolated and established as continuous lines. They will be inoculated into immunosuppressed mice to determine their malignant potential and their histologic cell type. Our primary aim, as stated above, is to show that mouse epithelial cells can be transformed into carcinoma cells in tissue culture. They would be very useful then for more extensive studies on the interplay of chemicals and virus in epithelial cell malignancies. Eventually, we hope such a line could be used to test the carcinogenic potential of various contaminants of our environment.

This interrelationship of C-type virus and carcinogens will be also assessed by using the C57/Black cell line (see p. 10). This epithelial-like cell line should be helpful since it cannot be transformed by standard concentrations of methyl cholanthrene and it is not making any C-type virus. Since we know C57/Black mice contain both ecotropic and X-tropic virus genomes (8,16), these cells will be treated with halogenated pyrimidines and tested for the induction of C-type virus. If a virus can be expressed after such treatment, the virus-producing cells will be exposed to methyl cholanthrene. These experiments will further substantiate whether the release of endogenous C-type virus from cells is a necessary prerequisite for carcinogenesis. In connection with these studies, the line will also be superinfected with a standard AKR type virus and then assayed for sensitivity to transformation by chemicals.

B. In vivo studies

Our initial experiments with the C57/Bl mice will be continued and should serve as a useful system for further in vivo attempts to elucidate a possible concerted role of endogenous viruses and chemicals in carcinogenesis. If we find chemical treatment results both in selective suppression of antibodies to the xenotropic virus and activation of the virus in the resulting carcinomas we have a provocative model for more detailed studies. While awaiting results in the initial experiments, we would like to begin animal studies aimed at uncovering genetic factors which may influence virus and chemical carcinogenesis. For these studies we must determine first the type of gene and/or number of genes which determine the expression of the endogenous xenotropic virus.

We know the NZB mouse has full expression of infectious virus in all its cells even in embryonic life (8,16). By full expression we mean that any cell established in culture from that animal actively produces relatively good titers of xenotropic virus. In contrast, cells from other strains are negative

or yield low titers of virus and only from certain organs (e.g. spleen). The NZB strain therefore, would be used for these genetic experiments. We have found, moreover, that crosses of NZB with C57/Black or NZW mice result in progeny mice which also have full expression of infectious xenotropic virus (8,16). Virus expression in NZB mice, then, resembles the expression of ecotropic virus in AKR mice. Studies similar to those carried out to understand the genetics of the AKR virus will be followed for elucidating the genes of xenotropic virus expression (23-25).

In collaboration with Dr. Kouri, NZB mice will be crossed with 129/J mice. This strain, although possessing antibody to X-tropic virus, has not yet yielded any infectious virus (Levy, unpublished observations). The virus must then be present in very low titers. Cells from the F_1 generation, like other NZB hybrids, should produce good titers of the virus. This will be ascertained by testing tissues for virus from certain animals from this generation. Then, by crossing the (NZBx129/J)F_1 generation back to 129/J parent we can determine whether the gene for X-tropic virus expression is single or multiple. If it is single we would expect cells from fifty percent of the backcross generation to produce significant titers of X-tropic virus. If multiple genes are involved, 75% or more will maintain this ability to make virus. In conjunction with the virus studies proposed, Dr. Kouri will treat the backcross progeny with methyl cholanthrene to ascertain their susceptibility to chemical carcinogens compared to the parental line. We know both NZB and 129/J animals have a diminished capacity for induction of AHH (26,27). For these reasons higher concentrations of the chemical (300-500 µg) will be used. We would like to show that tumor induction occurs primarily in those animals which also have the genetic capability of expressing xenotropic virus.

For these studies on cocarcinogenesis, a hemisplenectomy will be performed by Dr. Kouri (without sacrificing the animal) to provide information on the AHH inductibility of the mouse under study and its capacity for spontaneous or full expression of infectious virus. The AHH assays will be conducted as already described (26,27). For virus studies, the spleens will be minced and immediately plated on human foreskin cells pretreated with diethylaminoethyl-dextran to increase the sensitivity of the assay (28). After three passages, these cells will be cocultivated with the NRK-Harvey cells. Filtered seven day supernatants from these cocultivated cultures will be assayed for focus forming activity on human and rat cell cultures. If the NZB genome for virus expressions is present we would expect titers of X-tropic pseudotype sarcoma virus to be $>10^2$/cc.

Subsequent studies would deal with selecting strains of mice with different capacities for X-tropic virus expression and aryl hydrocarbon hydroxylase activity. The following strains are being considered:

	X-tropic virus expression (15)	Aryl-hydrocarbon induction (26,27)
NZB	+	-
129/J	-	-
C57/Black	+	+
C57/Black/10	-	+

In these experiments, the above strains will be treated with methylcholanthrene as described and the degree of tumor induction and activation

of X-tropic virus will be determined. In those shown with good AHH activity 150 µg of MC will be used; others will receive 500 µg. Virus studies will include cultivation of the tumor resulting from MC treatment and assaying it for both ecotropic and xenotropic virus production by standard techniques (8,16). Moreover, during all these studies, serum samples will be collected for determination of neutralizing antibody titers to ecotropic and xenotropic viruses.

C. Melanoma cells

We would like to continue studies on this interesting epithelial cell carcinoma. We want to determine whether one particular event in melanoma cells results in both virus activation and dedifferentiation as reflected by the decrease in pigment formation. Moreover, we would like to see if viruses, particularly the xenotropic virus, can be induced in this line by methyl cholanthrene or other hydrocarbons as well as halogenated pyrimidines and/or protein inhibitors (29). Finally, we would like to note if resumption of pigment formation (which occurs with certain cells of this cell line after IUDR has been removed) correlates with a decrease in virus production or more specifically in a selective change in the type of virus progeny being made. Finally, the loss of malignancy with virus activation will be evaluated. These studies are aimed at examining conditions by which differentiation, carcinogenesis, and virus expression are interrelated. Moreover, mechanisms by which malignant changes are maintained by the cell can be investigated.

D. Human xenotropic virus

Dr. Kouri is beginning a study directed at determining if human cells can be transformed by chemical hydrocarbons when microsomal fractions of mouse liver homogenates are used as a source of the AHH enzymes needed for production of the active chemical factors. Any transformed cells will be sent to us for evaluation for possible human xenotropic C-type virus. These studies will include:

1. Examination by electron microscopy.

2. Measurement of reverse transcriptase.

3. Cocultivation of the cells with a variety of animal cells which we are cultivating in our laboratory. A list of some of these cells and their growth capabilities is given in Table VI.

Significance

Theories on the role of viruses in cancer have suggested that a genetic message for a virogene and oncogene exist in all living cells (9). The discovery of the xenotropic virus has provided support of the virogene hypothesis, but the connection of this genetic message to oncogenes has not been established. Chemical carcinogenesis appears to be different from viral oncogenesis since chemicals require probably two steps for ultimate tumor induction (30). The experiments outlined in this proposal are aimed at elucidating the basic interplay between two factors, chemicals and viruses (particularly xenotropic viruses), in the possible production of carcinomas. They are designed to demonstrate that oncogenes may require

other determinants for expression than virogenes even if both are part of the same genetic molecule. The establishment of an epithelial line from the NZB liver, if confirmed by subsequent studies, represents the first true epithelial type cell line obtained from mice and promises to be very useful for testing various factors, particularly chemicals, for their carcinoma-inducing properties.

The role of the xenotropic virus has not been demonstrated. The cause of carcinogenesis (chemical or spontaneous) has not been determined and C-type viruses capable of producing epithelial cell tumors have not been isolated. It seems logical to us that viruses that are clearly endogenous to all members of a species may be responsible for common tumors which occur probably by interaction with environmental stimuli or common chemical contaminants. Since epithelial tumors represent the majority of cancers present in man, further studies on epithelial cell carcinogenesis and xenotropic viruses is important. The unraveling of this mystery may indeed bring us closer to the basic understanding of chemical carcinogenesis and the means by which we may prevent it.

12. Semi-Annual Progress Report

I. INTRODUCTION

Eighty percent of human cancers are carcinomas, and the great number of these occur in the lung. It is apparent to us that in vivo and in vitro systems directed at studying the induction of carcinogenesis in normal epithelial cells are important in trying to understand the variables which could be responsible for carcinoma production. Moreover, the investigation of carcinoma cells established in culture may help us understand the factors responsible for maintaining the malignant changes. For these reasons, our research has dealt primarily with three related topics.

A. Development of a model in vitro system for studying carcinoma induction.

B. Studies on the relationship of xenotropic viruses to chemical carcinogenesis.

C. Studies of carcinoma cells in vitro.

A. Development of a model in vitro system for studying carcinomas.

1. Background and Recent Results

Our initial research dealt with attempts to develop a model system in tissue culture for studying epithelial cell transformation. We chose the mouse system not only because of the readily available inbred strains, but also because it is a mammalian cell system which has endogenous RNA viruses that are well-understood and characterized. Tissue culture techniques would permit rapid methods for studying the possible concerted role of viruses and chemicals in epithelial cell malignancies. Moreover, once an epithelial line is established, various contaminants in our atmosphere can be checked for their carcinogenic potential.

In the course of this experimentation we learned that cell morphology in tissue culture is not an accurate method of distinguishing cell types (see Reports to the Council 1972, 1973). This conclusion is supported by the results of Sanford, et al. and others (1-3) who have noted that epithelial cells can change into fibroblast-like cells and vice versa. Based on our results with cultivated epithelial-like cells (see below) we have realized that the most accurate method for determining whether true epithelial cells have been cultured, and have been transformed, is the in vivo production of carcinomas in specific animal hosts. For this reason, our ultimate determination of epithelial cell histology is the induction of carcinomas in immunosuppressed mice.

We initially attempted to cultivate epithelial cells from mouse tracheas or skin by a variety of different techniques. All these attempts yielded islands of epithelial-like cells in culture but after a period of one to two weeks the surviving epithelial-like cells either spontaneously changed or they were replaced by fibroblast-like cells which grew rapidly to confluency. On occasion we observed that some islands grew and maintained characteristics of epithelial cells. Their cell division occurred only at the periphery and confluency required a period of one or two months. Petri dishes then became

filled with cells morphologically resembling epithelial cells. The same cells, however, when transformed by murine sarcoma viruses, or by the combined activity of chemicals and murine leukemia virus, only induced fibrosarcomas not carcinomas in immunosuppressed mice (Experiments conducted in collaboration with Dr. Paul Arnstein, Berkeley).

Our recent studies have dealt with established cell lines which have epithelial-like cell morphology.

a. NZB mouse epithelial-like cell line (C-1,S-2)

We have derived an epithelial-like cell line from NZB embryo cells. Since these cells produce xenotropic murine leukemia virus (see p. 11) they seemed ideal for studying directly the interaction of chemicals with endogenous C-type viruses. The line was tested for aryl hydrocarbon hydroxylase (AHH) by Dr. Richard Kouri (MBA, Bethesda) and was found to have low levels of the enzyme. This cell line (C-1,S-2) when treated with a small amount of methyl cholanthrene (MC) or benzo(a)pyrene produced foci of cell transformation which were easily recognized in the cell monolayer (see Fig. 4,5 of Annual Report, 1973). The cells therefore seemed to provide a good system for measuring potential carcinogenic compounds in vitro. To enhance the susceptibility of the cells to chemical transformation, pretreatment with non-carcinogenic hydrocarbons was tried. The results indicated, however, that pretreatment did not significantly increase the amount of focus formation induced by small amounts of MC (0.1 ug/cc). Moreover, foci isolated from this cell line and established in culture have produced only sarcomas in mice. These results indicated that either the cells when transformed reverted to a fibroblast morphology, or they originated from fibroblast cells with epithelial cell morphology. Recently these epithelial-like cells when maintained up to passage 9-12 spontaneously developed foci which did not appear to be any different from those induced by chemical alone, nor did they produce any different type of tumor in inoculated immunosuppressed NIH Swiss mice (Arnstein, personal communication). Such results, though telescoped in fewer passages with the NZB line, resembled the data published by Freeman, et al. (4-6). These authors found that the same rat cell line which could be transformed by the combined treatment with chemical and virus developed spontaneous foci of similar cell alteration after numerous passages. Since spontaneous foci occurred at early passages in this NZB cell line and the transformed cells only produced sarcomas, we decided to abandon the use of this cell line and attempt similar experiments with other epithelial-like cell lines established in culture.

2. C57/Black mouse epithelial-like cell line

We have derived from the peritoneum of a C57/Bl mouse, an epithelial-like cell culture, which is not releasing any xenotropic virus. When exposed to various concentrations of methyl cholanthrene, higher levels of the chemical were needed for toxicity (5 ug/cc) than seen with the NZB embryo cell line, and no transformation occurred. Cells remaining in the cultures which received toxic doses of MC were maintained with the hope that transformation would be noted. However, these surviving cells resumed their epithelial morphology and no transformation occurred after 10 passages. This line will be further evaluated for its ability to be transformed after superinfection with standard MLV or after induction of X-tropic virus (see p. 5).

3. A liver cell line from an NZB mouse has been established. It has an epithelial-like cell morphology; it is discussed in the next section (see below).

B. The relationship of xenotropic virus to chemical carcinogenesis

1. Background

Four years ago in a study of NZB mice we were able to demonstrate the presence of a C-type RNA tumor virus which has an unusual tropism (7). Instead of being infectious for cells from its own animal species, it could only be propagated in cells from animals of different species; for this reason it has been called a xenotropic (X-tropic) virus (8). We have been able to isolate this virus from several mouse species and believe it is a common endogenous virus of mice (Table 1). Moreover, sera from most if not all mouse strains, neutralize the virus at titers of at least 1:100. NZB mice and their hybrids usually have titers of 1:1000 and occasionally titers of 1:10,000 have been detected (Levy, manuscript in preparation) (Table II).

The role of this mouse xenotropic virus has not yet been determined. Because many chemically induced tumors do not yield any standard mouse C-type virus and yet have CF antigen, we decided that these tumors should be examined for the presence of xenotropic virus. There was the possibility as suggested by Huebner, et al. (9-12) that the interaction of chemical with endogenous virus could result in carcinogenesis. Both in vitro and in vivo studies have been begun aimed at elucidating this theory.

a. In vitro studies

We believe the most efficient method of looking at the induction of carcinoma by the interaction of chemical and virus is by in vitro testing. As noted above, we have attempted to grow cells from epithelial tissues and test their susceptibility to chemical and/or virus transformation. We were especially interested in developing a line from NZB mice since these cells would be ideal for studying direct interactions of chemical and endogenous C-type virus.

Our initial efforts to establish an epithelial cell line from NZB cultures resulted in the establishment of the C-1,S-2 clonal cell line. Although epithelial-looking morphologically, this line, after transformation, only produces sarcomas in mice and so its value as an in vitro model of carcinomas was diminished.

From further studies on other lines derived from NZB mice and their hybrids a line from an NZB liver culture has been established. These cells grow in a very unusual pattern. Instead of assuming whorls or parallel accumulations of fibroblast-like cells, these "liver" cells maintain a pavement-like appearance and grow to confluency without significant piling up (Fig. 1). The most interesting feature of these cells is their pattern of growth which leaves areas free of cells scattered throughout the monolayer (see Fig. 2). The picture suggests liver parenchymal cells allowing spaces for the in-growth of blood vessels or bile canaliculi.

One-cell clones from the established line (3105) continue to maintain this unusual morphologic characteristic. Using standard techniques (7,8) we have determined that this line, as have all cells from NZB mice, produces xenotropic C-type virus. The cells were cocultivated with the NRK-Harvey

cell line, a non-virus producing rat cell line transformed by the Harvey sarcoma virus (13). After seven days, the supernatant fluids from the cocultivated cultures were tested for focus activity in human cells and significant amounts of pseudotype sarcoma virus were detected. The line, therefore, if epithelial would permit us to conduct in vitro studies on the possible interplay of endogenous C-type viruses and chemicals in carcinogenesis. Before initiating any extensive studies on this line, we did not want to be faced with the same problem presented by the C-1,S-2 line, i.e. epithelial-looking cells which may be fibroblasts. The "liver" line offered some advantage over the other epithelial-like cells since if epithelial, it should be making liver enzymes.

The cells have been examined by Dr. Urs Meyer in Dr. R. Schmidt's laboratory for the presence of the liver enzyme, delta-levulenic acid synthetase but were negative. Such a result, however, is not unusual for liver cells established in culture from other species (Meyer, personal communication). They have been tested by Dr. Ulrich Gehring in Dr. Tomkins' laboratory for the presence of tyrosine aminotransferase enzyme. The first set of experiments have been very encouraging. Enzyme activity, though at a low level, was detected in the cultures and this activity was increased three-fold by treating with dexamethasone. The cells, therefore, behave similarly to the rat hepatoma cell line (HTC) which Tomkins, et al. use in dexamethazone induction studies (14). Dr. Gehring believes the data suggest 3105 is a mouse liver line and is doing further studies on it to confirm these initial results. The line is also being evaluated for the presence of the liver enzymes, glucose-6-phosphatase and B-hydroxy-B-methyl glutaryl coenzyme A reductase (15) by Dr. M. Siperstein of the Department of Medicine.

If these tests confirm the fact that the cells are liver parenchymal cells, we shall proceed with standard techniques for studying chemical transformation in vitro. This line has been inoculated into antithymocyte serum (ATS) treated NIH Swiss mice and nude mice. No tumors have appeared after 3 months (Arnstein, personal communication). This suggests that the line has not spontaneously transformed.

In vivo studies

In collaboration with Dr. Richard Kouri (MBA, Bethesda) a prospective study has been begun of chemical carcinogenesis in inbred strains of mice. The C57/Black strain was chosen for the first experiment since it has xenotropic virus as well as mouse-tropic (ecotropic) virus (8,16) so we could assess to some extent the role of both these viruses in carcinogenesis. We know that sera from C57/Black mice generally can neutralize xenotropic pseudotype sarcoma virus at titers of 1:100-1:300 (Table II); sometimes titers as high as 1:1000 are observed. We are interested in determining whether treatment of C57/Black mice with methyl cholanthrene decreases their antibody titer to xenotropic virus and permits the emergence of endogenous X-tropic virus and the ultimate production of carcinomas. For our initial study, 25 C57/Bl mice were treated with MC diluted in triocane. Twenty-five control mice received only the vehicle. Blood samples were taken prior to treatment and will be taken every three weeks after treatment until the development of carcinomas. In some animals these carcinomas will be removed and the animals maintained for another 3 months. In preliminary studies on four C57/Black mice already carrying chemically induced tumors the xenotropic virus was demonstrated in two out of the four cases (Table III). In two tumors, a B-tropic ecotropic virus was isolated. This high frequency of recovery of B-tropic virus was surprising and may reflect the increased sensitivity of

our assays which include passage of indicator mouse and human cells for three weeks before testing them for ecotropic and/or xenotropic virus.

We are now three months into this study of chemically induced tumors and the results on the mouse sera selected from the 50 mice in this study are given in Table IV. They indicate that antibody titers to the virus are present before treatment with the chemical and range between 1:100 and 1:1000. In a preliminary survey of some sera obtained 2-7 weeks after MC treatment, the titer of antibody to the X-tropic virus was found to be diminished. We shall compare these results with the control and vehicle-treated mice to determine if they are significant. We are encouraged, however, by these first observations since antibody studies in our laboratory with other C57/Black mice do not show a drop in titer particularly to levels less than 1:10. These results suggest some relationship may exist between chemical exposure, virus activation without virus neutralizing antibody response, and the ultimate development of malignancy.

C. Studies of carcinoma cells in vitro: C57/Bl melanoma cell line

We are collaborating with Dr. Selma Silagi (New York) in studies directed at determining factors which induce or maintain carcinogenesis. Dr. Silagi has derived from C57/Bl mice a continuous line of malignant melanoma cells which produce pigment in tissue culture (17). After the addition of halogenated pyrimidines, however, pigment formation ceases, the cells begin to produce C-type virus, and they become less malignant for animal hosts (18,19). We have confirmed the results on virus activation in our laboratory. Initially experiments were conducted in which melanoma cells were cocultivated for three weeks in the presence of NIH Swiss mouse embryo, BALB/c, mouse embryo and human foreskin cells. Following these procedures, the mouse cells were cocultivated with XC cells to detect mouse-tropic (ecotropic) virus and the human cells were cocultivated with NRK-Harvey cells to detect the presence of xenotropic virus.

In these studies no virus could be detected in the melanoma cell line itself, even after long-term cultivation with the indicator lines. Nevertheless, within two days after 100 ug/cc of iododeoxyuridine (IUDR) was added to the culture, pigment formation decreased and virus production could be detected. The viruses thus far isolated have been of two types, N-tropic and B-tropic (20). (Table V) No X-tropic virus has yet been detected. The recovery of B-tropic virus is unusual since most latent viruses activated in tissue culture from B-type cells are N-tropic (21,22). Certain lines of these melanoma cells have spontaneously begun to release B-tropic virus and they produce less pigment than the non-virus producing melanoma cells. It appears then that dedifferentiation or decreased pigment formation occurs at the time of C-type virus production. These cells provide an excellent opportunity to look at the interplay of differentiation, virus activation and epithelial cell carcinogenesis.

TABLE I

RECOVERY OF XENOTROPIC VIRUSES FROM VARIOUS MOUSE STRAINS

MOUSE STRAINS	POSITIVE/TOTAL	RANGE IN VIRUS TITER (FFU)/cc
NZB	42/42	1-1000
(NZBxNZW)F_1	8/8	1-1000
(NZBxC57/Bl)F_1	4/4	1-200
NZW	2/2	1-1000
C57/Black	5/8	1-1000
NIH Swiss	12/16	1-1000
C57/Leaden	1/1	1-50
BALB/c	1/2	1-20
Nude	2/2	1-20
C57/Black 10 (Snell)	2/2	1-20
C57/Black 10 (58N)	2/2	1-20
SJL/J	0/5	-
SWR	0/2	-
129/J	0/2	-
A/J	0/2	-

[1]Spleen, thymus, and kidney from each adult animal was assayed for infectious xenotropic virus by standard technique. Recovery of the virus from one of these organs is registered as a positive.

[2]Number of infectious pseudotype sarcoma virus particles produced after cocultivation of cells from these mice with non-virus producing MSV-transformed rat cells ( ). The titer was determined by focus forming activity (FFU) in human or rat cells. The number gives a general estimate of the amount of infectious xenotropic virus being produced by the cultured cells.

TABLE II

NEUTRALIZATION OF XENOTROPIC VIRUS BY SERA FROM VARIOUS MOUSE STRAINS

STRAIN	NUMBER OF ANIMALS TESTED	AVERAGE TITER[1]
NZB	8	1000-10,000
(NZBxNZW)F_1	8	1000-4000
NZW	3	100-200
C57/Black	15	100-200
NIH Swiss	23	10-100
C57/Leaden	9	100-200
SJL/J	3	100-200

1. Neutralization titer was determined by the neutralization of the focus forming activity of xenotropic pseudotype sarcoma virus.

TABLE III

RECOVERY OF MLV FROM CHEMICALLY-INDUCED TUMORS IN C57/BLACK MICE

	ANIMAL	X-TROPIC	E-TROPIC
1.	TUMOR	+	0
	SPLEEN	0	0
2.	TUMOR	0	0
	SPLEEN	+	0
3.	TUMOR	0	0
	SPLEEN	+	+
4.	TUMOR	+	+
	SPLEEN	+	+

[1]Four C57/Black mice were inoculated subcutaneously with 3-methyl-cholanthrene. Tumors developed after 3 months at the site of inoculation. These animals were sacrificed and their tumors and spleens were cultivated and tested for the presence of xenotropic (X-tropic) and ecotropic (E-tropic) murine leukemia viruses (MLV).

[2]All ecotropic isolates were of the B-tropic subgroup of MLV.

TABLE IV

EFFECT OF METHYL-CHOLANTHRENE (MC) ON SERUM ANTIBODIES TO THE MURINE XENOTROPIC VIRUS[1]

Animal No.	Before Treatment	2 Weeks After MC Treatment	4 Weeks After MC Treatment	7 Weeks After MC Treatment
472	100	-	100	10
473	100	-	-	
474	1000	10	10	10
475	1000	-	-	
476	1000	-	<10	<10
477	100	-	-	
478	1000	-	-	
482	-	10	10	<10
484	-	10	10	<10
487	-	-	10	10
494	-	-	10	-
495	-	-	10	-
496	-	-	100	<10

[1]Numbers represent the serum dilution which gave at least 66% neutralization of focus formation by NZB pseudotype sarcoma virus.

TABLE V

RECOVERY OF C-TYPE VIRUSES FROM CULTURED C57/BLACK MELANOMA CELLS

Cells	Treatment	Virus Recovered
Melanoma	0	0
↓	IUDR alone	B-tropic AKR type
	IUDR + BALB-ME	B-tropic AKR type
	IUDR + NIH-ME	N-tropic AKR type
	IUDR + HuF	0

Cells were exposed to iododeoxyuridine (IUDR), 100 µg/cc for 36 h. Cultures were then washed and refed with maintenance media. To certain treated cultures, BALB/c or NIH Swiss mouse embryo (ME) or human foreskin (HuF) cells were added. After 7 days, the fluids were collected and titered on NIH-ME and BALB/c ME. The cultures receiving HuF cells were passed three times and then cocultivated with NRK-Harvey cells. The 7-day supernatant from that culture was tested for X-tropic pseudotype sarcoma virus by focus formation assay on human cells.

TABLE VI

ANIMAL CELL LINES PRESENTLY CULTIVATED IN THE LABORATORY

Lines Available	Growth Characteristics
Mammalian:	
Anteater	Large, fat fibroblast, very granular, moderate growth rate.
Bat lung	Small cells, epithelioid, very rapid growth.
Bear lung	Large fibroblast cell, rapid growth rate.
Bovine embryo kidney	Fibroblast cell, moderate growth rate.
Deer kidney	Fibroblast, fairly rapid growth.
Dog kidney	Epithelioid, very rapid growth (similar to bat lung.
Gazelle lung	Fibroblastic cells, fairly rapid growth rate.
Chinese hamster embryo	Fibroblastic cells, moderate growth rate.
Chinese hamster thymus	Fibroblastic cells, moderate growth rate.
Syrian hamster embryo	Fibroblastic cells, moderate growth rate.
Lion lymph node	Large fibroblast, moderate growth rate.
Embryonic rhesus monkey heart	Fibroblastic cells, moderate growth rate.
African water mongoose	Large fibroblast, moderate growth rate.
Peccary kidney	Very vacuolated granular epithelial-like cells; rapid growth.
Racoon uterus	Fibroblastic cell; moderate growth rate.
Avian:	
White Longhorn chicken	Fibroblastic cells, moderate growth rate.
Peking duck	Fibroblastic cells, moderate growth rate.
Red-necked pheasant	Fibroblastic cells, moderate growth rate.
Golden-necked pheasant	Fibroblastic cells, moderate growth rate.
Quail	Fibroblastic cells, moderate growth rate.
Insect:	
Mosquito Chao A	Epithelial, vacuolated cells, very fast growth at room temperature.
Aedes albopictus	Epithelial, vacuolated cells; very fast growth at room temperature.

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LEGENDS TO FIGURES

Figure 1. Culture of epithelial-like cells derived from an NZB liver. Note the cells pavement-like appearance of the rectangular-shaped cells. X100.

Figure 2. Culture of epithelial-like cells derived from an NZB liver. Note the spaces free of cells. It gives the appearance of liver parenchymal cells allowing room for the ingrowth of blood vessels or bile canaliculi. X40.

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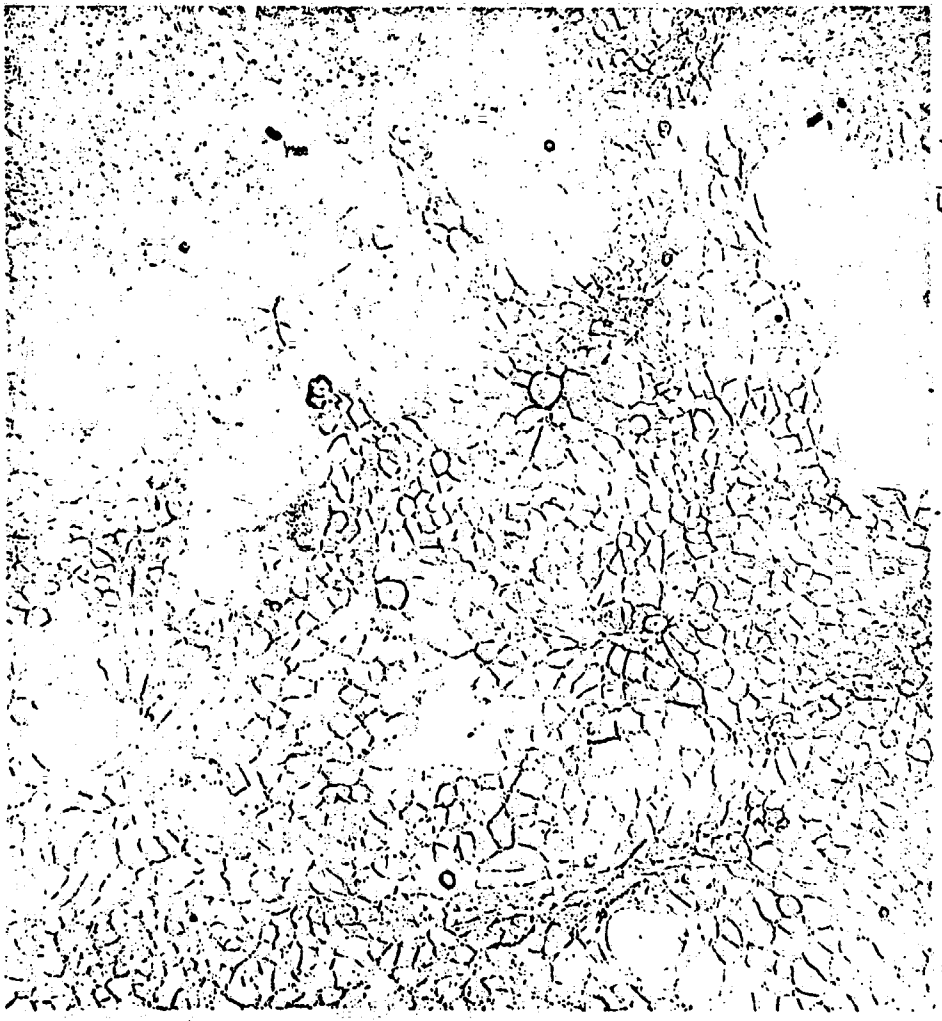

Fig 1

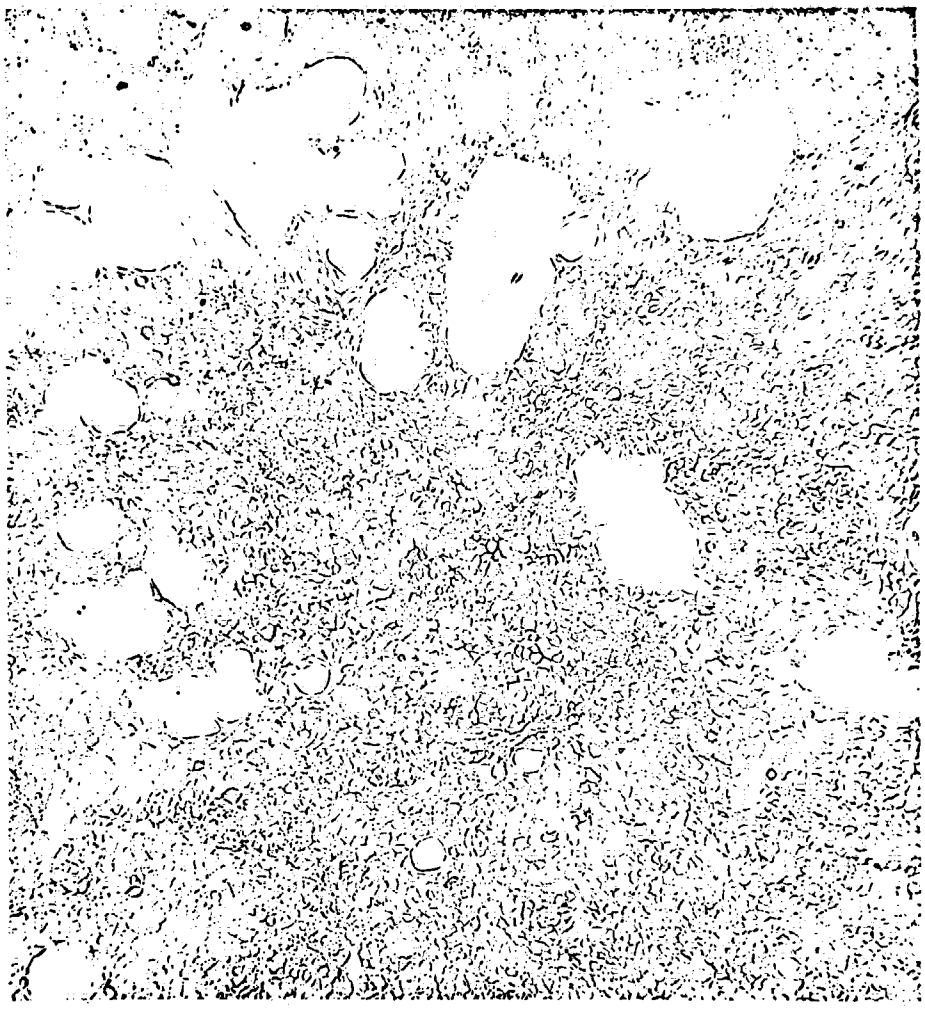

Fig 2